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A G-Quadruplex-Interactive Potent Small-Molecule Inhibitor of Telomerase Exhibiting in Vitro and in Vivo Antitumor Activity

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ABSTRACT

The telomerase complex is responsible for telomere maintenance and represents a promising cancer therapeutic target. We describe herein the antitelomerase and antitumor properties of a small-molecule compound designed by computer modeling to interact with and stabilize human G-quadruplex DNA, a structure that may form with telomeric DNA, thereby inhibiting access to telomerase. The 3,6,9-trisubstituted acridine 9-[4-(*N*,*N*-dimethylamino)phenylamino]-3,6-bis(3-pyrrolodinopropionamido) acridine (BRACO19) represents one of the most potent cell-free inhibitors of human telomerase yet described (50% inhibitory concentration of 115 ± 18 nM). Moreover, in contrast to G-quadruplex interactive agents described previously, BRACO19 did not cause nonspecific acute cytotoxicity at similar concentrations to those required to completely inhibit telomerase activity. There exists a 90-fold differential (mean 50% inhibitory concentration for acute cell kill across seven human tumor cell lines of 10.6 ± 0.7 μM). The exposure of 21NT human breast cancer cells, which possess relatively short telomeres, to nonacute cytotoxic concentrations of BRACO19 (2 μM) resulted in a marked reduction in cell growth after only 15 days. This was concomitant with a reduction in intracellular telomerase activity and onset of senescence as indicated by an increase in the number of β-galactosidase positive-staining cells. Intraperitoneal administration of nontoxic doses of BRACO19 (2 mg/kg) to mice bearing advanced stage A431 human vulval carcinoma subcutaneous xenografts and previously treated with paclitaxel induced a significant increase in antitumor effect compared with that observed with paclitaxel alone. BRACO19 thus represents the first of a "second generation" of G-quadruplex-mediated telomerase/telomere-interactive compounds. It possesses nanomolar potency against telomerase but low nonspecific cytotoxicity, growth inhibitory effects, and induction of senescence in a human breast cancer cell line and, moreover, significant antitumor activity in vivo when administered post paclitaxel to mice bearing a human tumor xenograft carcinoma.

One of the recognized acquired capabilities of cancer is a limitless replicative potential (Hanahan and Weinberg, 2000). It is now evident that this ability relates to the maintenance of telomeres, tandem repeated DNA sequences ($[TTAGGG]_n$ in humans) at the ends of chromosomes with associated proteins. Telomeres protect the ends of chromosomes from recombination, fusion, or being recognized as damaged DNA and need to be maintained above a critical length and or in a capped status, otherwise cellular crisis ensues (Blackburn, 2000, 2001). In normal cells, approximately 100 bases of telomeric DNA is lost at every cell division, due to the "end-replication" problem, the inability of DNA polymerase to fully replicate the ends (Harley et al., 1990). However, in cancer cells, telomere length is stably maintained, generally at relatively short lengths compared with normal cells. In approximately 85% of tumors telomere length is maintained by the telomerase complex, which includes a specialized reverse transcriptase ribonucleoprotein that synthesizes the G-rich strand of telomeres by adding single-stranded TTAGGG repeats (Kim et al., 1994; Holt and Shay, 1999). In a small percentage of tumors, an alternate pathway for telomere length may be operational and involves recombination events (Bryan et al., 1997; Dunham et al., 2000).

The associated activity of telomerase in the majority of tumors combined with its absence in most adult normal tissues has generated considerable interest in targeting the enzyme and associated telomeres in a cancer therapeutic context (Neidle and Kelland, 1999; Pitts and Corey, 1999; Bearss et al., 2000; Kelland, 2000). Furthermore, high telomerase expression has been correlated with a poor prognosis in some tumor types, notably neuroblastoma (Hiyama et al., 1995) and breast cancer (Clark et al., 1997). Telomerase is

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ABBREVIATIONS: SRB, sulforhodamine B; TRAP, telomeric repeat amplification protocol; PCR, polymerase chain reaction; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

composed of a catalytic subunit (hTERT, hTRT) (Meyerson et al., 1997), an RNA domain (hTR) (Feng et al., 1995), and a further protein (TEP-1) (Harrington et al., 1997). In turn, various proteins such as TRF1 (van Steensel and de Lange, 1997), TRF2 (van Steensel et al., 1998), tankyrase (Smith et al., 1998), Ku (Hsu et al., 1999), TIN2 (Kim et al., 2000), and Pot-1 (Baumann and Cech, 2001) have been shown to associate with telomeres.

Several genetic (by using transfection of dominant negative forms of hTERT; Hahn et al., 1999; Zhang et al., 1999) and pharmacological (including use of antisense molecules; Herbert et al., 1999) target validation studies all concur in suggesting that inhibitors of the telomerase/telomere machinery might confer clinical antitumor efficacy. In particular, two independent studies involving the transfection of dominant-negative mutants of hTERT into tumor cell lines resulted in cell death by apoptosis and loss of tumorigenicity (Hahn et al., 1999; Zhang et al., 1999). The onset of death correlated with initial telomere length, with tumor cells possessing short telomeres dying before those with longer telomeres. Our approach has been to focus on the rational discovery of small-molecule telomerase inhibitors that interact with the telomeric G-rich overhang rather than the enzyme itself, and that would have "drug-like" features. Telomeric DNA is capable of folding into four-stranded guanine quadruplex (G4) structures, including in the macronuclei of *Stylonychia lemnae* (Schaffitzel et al., 2001), which then inhibits telomere elongation by telomerase (Zahler et al., 1991). This has led to a rational search for small molecules that can selectively interact with and stabilize G-quadruplexes (for reviews, see Mergny and Helene, 1998; Kerwin, 2000). After our joint original discovery of tricyclic anthraquinone-based G-quadruplex-interactive telomerase inhibitors (Sun et al., 1997; Perry et al., 1998a,b), a number of other compound classes have been identified, including fluorenones (Perry et al., 1999b), bisubstituted acridines (Harrison et al., 1999), cationic porphyrins (Wheelhouse et al., 1998; Izbicka et al., 1999), a perylenetetracarboxylic diimide derivative (Fedoroff et al., 1998), indolo-quinolines (Caprio et al., 2000), and a benzonaphthofurandione tetracyclic compound (Perry et al., 1999a). However, a severe limitation thus far with these compounds is that they are not particularly potent telomerase inhibitors (with 50% inhibitory concentrations in cell-free assays of greater than 1 μM) and that they possess relatively poor selectivity for binding to quadruplex versus duplex DNA. This is reflected in these compounds showing acute cell kill at concentrations similar to those required for telomerase inhibition. Recently, some other series of potent G-quadruplex-interactive telomerase inhibitors based on ethidium (Koeppel et al., 2001) dibenzophenanthrolines (Mergny et al., 2001), pentacyclic acridines (Gowan et al., 2001), and the microbial product telomestatin (Shin-ya et al., 2001) have been described but, with the exception of our pentacyclic acridine, no cellular (cytotoxicity) data were reported.

The aim of this study was to evaluate the telomerase inhibitory activity and in vitro and in vivo antitumor properties of a novel "second-generation" 3,6,9-trisubstituted acridine compound (BRACO19). The compound has been rationally designed using a molecular modeling approach developed initially with disubstituted amidoanthracene, 9–10 diones, and 3,6-disubstituted acridines (Read et al., 1999) to exploit the unique structural features of G-quadruplex DNA (Read et al., 2001). BRACO19 has been evaluated according to an established cascade as described for the above-mentioned compounds (Kelland, 2001), comprising an initial comparison of cell-free telomerase inhibitory activity in the TRAP assay (Kim et al., 1994) versus acute cytotoxicity against a small panel of human cancer cell lines (Perry et al., 1998a,b, 1999b; Harrison et al., 1999). The study reported herein has focused on growth inhibitory effects in a human breast cancer cell line by using long-term exposure to non-acute cytotoxic concentrations of agent. Finally, the antitumor activity of BRACO19 has been determined in vivo by using the A431 human vulval carcinoma xenografted subcutaneously onto immune-suppressed mice.

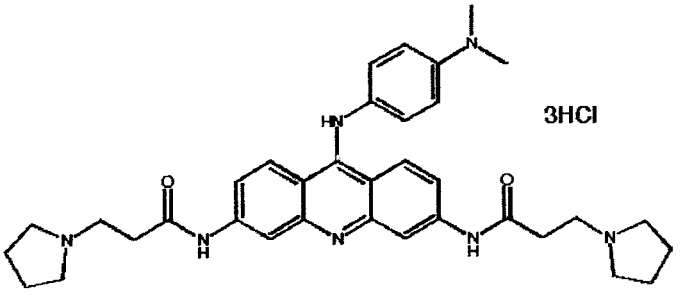
Materials and Methods

Reagents and Cell Lines

Chemicals were from Sigma Chemical (Poole, Dorset, UK) unless otherwise indicated. The synthesis and quadruplex versus duplex DNA binding properties of BRACO19 (Fig. 1) have been described recently (Read et al., 2001). The human breast cancer cell line 21NT (Cuthbert et al., 1999) was kindly provided by Dr. R. Newbold (University of Brunel, Brunel, UK) and was selected for initial whole cell studies because it possesses relatively short telomeres (Cuthbert et al., 1999). Other human cancer cell lines used were A431 human vulval carcinoma cells (that also possess relatively short telomeres; Zhang et al., 1999) and A2780, A2780cisR (possessing acquired drug resistance to cisplatin) SKOV-3, CH1, and CH1cisR (possessing acquired resistance to cisplatin) human ovarian cells (Raynaud et al., 1997). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal calf serum (Invitrogen), 0.5 μg ml^{-1} hydrocortisone, and 2 mM L-glutamine in a humidified 6% CO_2, 94% air atmosphere. For 21NT cells, 1 μg ml^{-1} insulin and 12.5 ng ml^{-1} epidermal growth factor were also added.

Growth Inhibition Assay

Acute growth inhibitory effects were assessed using the sulforhodamine B assay as described previously by us (Raynaud et al., 1997). Briefly, between 3000 and 6000 cells/well were seeded into 96-well microtiter plates and allowed to attach overnight. Drug (freshly dissolved at a concentration of 500 μM in water) was then added at a range of concentrations to quadruplicate wells and left in contact for 4 days. At this point, cell numbers were compared in treated versus control wells by fixing in ice-cold 10% w/v trichloroacetic acid (30 min) and staining with 0.4% SRB in 1% v/v acetic acid (15 min). Mean absorbance at 540 nm for each drug concentration was expressed as a percentage of the control untreated well absorbance.



BRACO19

Fig. 1. Chemical structure of BRACO19.

***Taq* Polymerase Assay.** As discussed previously (Neidle and Kelland, 1999; Kelland, 2000), because the TRAP (see below) for assessing inhibitory activity against telomerase is PCR-based, it was essential to ensure that positive inhibition in this assay is mediated through effects on telomerase rather than via nonspecific inhibition of the PCR. Therefore, test compounds were added at concentrations of 1, 5, 10, and 20 μM to a PCR 50-μl master mix containing 10 ng of pCI-neo mammalian expression vector (Promega, Southampton, UK) and forward (GGAGTTCCGCGTTACATAAC) and reverse (GTCTGCTCGAAGCATTAACC) primers (200 nmol) as described previously (Perry et al., 1999b). The product of approximately 1 kb was visualized using ethidium bromide after separation by electrophoresis on a 2% w/v agarose gel after amplification (30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min) by using an MBS thermal cycler (Thermo Hybaid, Ashford, Middlesex, UK).

TRAP. The ability of the trisubstituted acridines to inhibit telomerase in a cell-free assay was assessed using the TRAP as described previously (Perry et al., 1998a; Harrison et al., 1999). Telomerase was prepared from extracts of exponentially growing A2780 cells by lysing for 30 min on ice in a CHAPS-based buffer (0.5% w/w CHAPS, 10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, and 10% v/v glycerol) with 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride freshly added. The lysate was then centrifuged at 12,000 rpm for 30 min at 4°C, the supernatant collected, and stored frozen in aliquots at −80°C for up to 3 months. Total cellular protein was then determined and the TRAP performed in two steps by using 40 ng of protein. Step 1: telomerase mediated extension of a nontelomeric oligonucleotide forward TS primer (5′-AATCCGTCGAGCAGAGTT) (0.1 μg) contained in a 40-μl reaction mix comprising TRAP buffer (20 mM Tris-HCl pH 8.3, 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and 0.05% Tween 20), 0.05 μg of bovine serum albumin, 50 μM each deoxynucleotide triphosphate, and 3 μCi of [α-^{32}P]dCTP (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Protein was then incubated with the reaction mix, with or without compound at various final concentrations (well below that causing nonspecific inhibition of the PCR), for 20 min at 25°C. A lysis buffer (no protein) control, heat-inactivated (85°C for 10 min) protein control, and 25% protein control (10 ng) were included in each experiment. Step 2: Samples were heated at 85°C for 5 min to inactivate telomerase and during this time 0.1 μg of reverse CX primer (3′-AATCCCATTCCCATTCCCATTCCC) and 2 units of *Taq* DNA polymerase ("red hot"; Advanced Biotechnologies, Columbia, MD) were added. Oligonucleotide primers were obtained from Oswel Ltd. (Southampton, UK). A three-step PCR was then performed: 31 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. Telomerase-extended PCR products, with or without test compound, were then measured either by electrophoretic separation (8% w/w acrylamide denaturing gels, Sequagel; National Diagnostics, Atlanta, GA) and visualization by phosphorimaging or by harvesting on Whatman filters (25 mm) by trichloroacetic acid precipitation and then analyzing by liquid scintillation counting.

Long-Term Exposure Studies. Cells were grown in T80 tissue culture flasks at 1.25×10^5/flask and exposed to a nonacute cytotoxic concentration of BRACO19 (2 μM) or an equivalent volume of water (drug vehicle control) every 3 to 4 days. Every 7 days, the cells in control and drug-exposed flasks were trypsinized and counted using a hematocytometer and flasks reseeded with 1.25×10^5 cells. Remaining cells were collected and used for measurements described below. This weekly process, with twice-weekly drug addition, was continued until such time that there were fewer than 1.25×10^5 cells available for reseeding.

For measurements of telomerase activity within treated or control cells, protein extracts were prepared as described above for the TRAP and then known amounts of protein included in the above-described TRAP.

Measurements of Telomere Length. An approximation of telomere length was obtained using slot blotting from cell pellets collected weekly within the long-term exposure experiments. Pellets were washed in phosphate-buffered saline and DNA extracted using QIAamp blood kit (QIAGEN, Valencia, CA) as described by the manufacturer's instructions. DNA content was determined using a Genequant (Amersham Biosciences UK, Ltd.) and 25 and 50 ng from control or treated cells was loaded onto a Hybond-XL nylon membranes (Amersham Biosciences UK, Ltd.). Membranes were air-dried, denatured (using 0.5 M NaOH and 1.5 M NaCl), neutralized (0.5 M Tris-HCl pH 8 and 1.5 M NaCl), and cross-linked (Stratalinker; Stratagene, La Jolla, CA). Probes for telomere length (TTAGGGTTAGGGTTAGGGTTAGGG) and for centromeric loading controls (GTTTGAAACACTCTTTTTGTAGAATCTGC) were end-labeled using [γ-^{32}P]ATP. Slot blots were prehybridized with RapidHyb (Amersham Biosciences UK, Ltd.) for 30 min, denatured probes were added, and hybridized at 42°C for 3 h. Blots were exposed to phosphorimager screen for 1 h and visualized using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Blots were then stripped and checked clean by phosphorimaging and then hybridized with the centromeric probe as for the telomere probe. Image analysis was performed using ImageQuant software (Molecular Dynamics).

β-Galactosidase Staining

Cells (1.25×10^5) were plated out weekly from the above-described long-term exposure experiment into each well of a six-well plate and left overnight. Cells were then stained for β-galactosidase expression as a biomarker of cell senescence (Dimri et al., 1995) by using a β-galactosidase staining kit (Invitrogen BV, Breda, The Netherlands) according to the manufacturer's instructions. Positive-stained cells and the total number of cells per well were counted blind by an independent assessor. Positive cells were expressed as a percentage of total number per well.

In Vivo Antitumor Efficacy

Initially, the maximum-tolerated dose of BRACO19 was determined in NCr nude mice after single intraperitoneal injection of compound made up in 5% DMSO/95% water. Antitumor activity was then assessed using advanced stage A431 human vulval tumor xenografts, another tumor line shown to possess relatively short telomeres (Zhang et al., 1999). Approximately 2-mm^2 fragments of A431 tumor were implanted into adult female NCr nude mice, by trochar, subcutaneously in the flank under halothane anesthesia. Once palpable (approximately 6–8 mm in diameter) animals were randomized (five to six animals) into control or treatment groups and therapy started (day 0). The antitumor efficacy of BRACO19 was compared when administered alone (in 5% DMSO/95% water; 2 mg/kg i.p. daily for 5 days for 4 weeks from day 0) or post paclitaxel-based cytotoxic chemotherapy. Paclitaxel (ethanol/polyethoxylated castor oil; Bristol-Myers Squibb Co., Stamford, CT) was administered at 25 mg/kg i.p. on days 0, 4, and 8 either alone or followed by BRACO19 from day 13 (2 mg/kg i.p. on days 13–17, 20–24, 27–31, and 34–38). Mice were weighed and tumor volumes were determined by caliper measurements twice weekly from day 0. Tumor volumes were determined using the formula volume = $a \times b^2 \times \pi/6$ where a and b are orthogonal tumor diameters. Results are expressed as relative tumor volumes. Compound effectiveness was determined in terms of a treated/control volume ratio at particular days post the start of treatment.

All animal procedures were performed within the guidelines set out by the Institute of Cancer Research Animal Ethics Committee and the United Kingdom Coordinating

Committee on Cancer Research Committee on the Welfare of Animals in Experimental Neoplasia (Workman et al., 1999).

Statistical Analyses

Where appropriate, significance was tested using a Student's t test (two-tailed, unpaired). A P value of < 0.05 was considered significant.

Results

BRACO19 exhibited potent inhibition of telomerase in the cell-free TRAP (representative gel in Fig. 2A) with a mean 50% inhibitory concentration of 115 ± 18 nM (as derived from the dose-response curve shown in Fig. 2C, by using products harvested on filters). There was virtually complete inhibition of the formation of telomerase-induced telomeric hexanucleotide repeats at a concentration of only 500 nM.

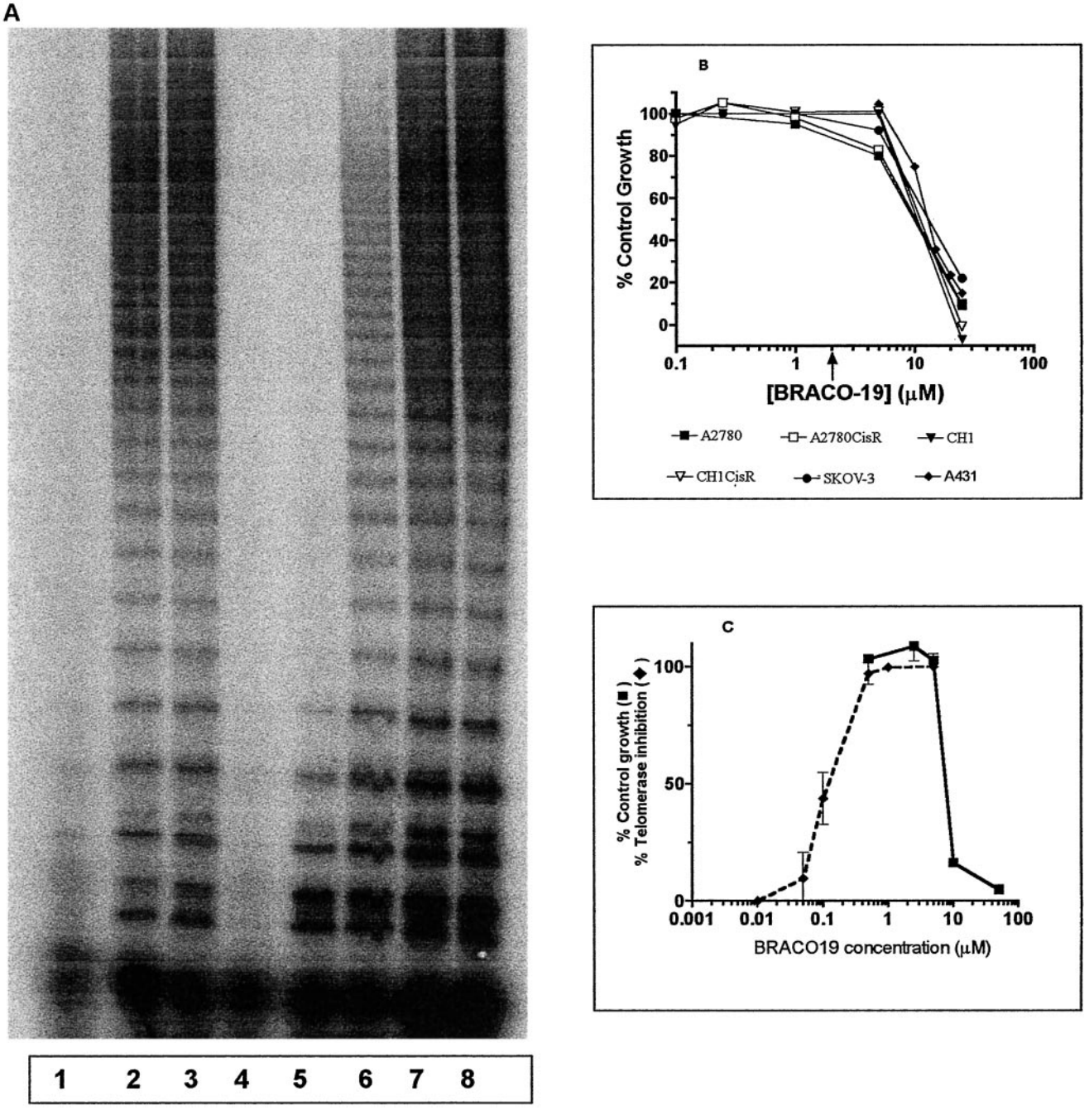


Fig. 2. A, inhibition of telomerase by BRACO19 in vitro by using TRAP. Lane 1, lysis buffer control; lane 2, A2780 extract, 10 ng of protein; lane 3, A2780 extract, 40 ng of protein; lane 4, 40 ng of A2780 extract heat-inactivated; lane 5, 40 ng of A2780 extract plus 0.5 μM BRACO19; lane 6, 40 ng of extract plus 0.2 μM BRACO19; lane 7, 40 ng of extract plus 0.1 μM BRACO19; and lane 8, 40 ng of extract plus 0.05 μM BRACO19. B, acute growth inhibition by BRACO19 (4-day exposure; SRB assay) in human ovarian carcinoma cell lines A2780 (■), A2780cisR (□), CH1 (▼), CH1cisR (▽), SKOV-3 (●), and human vulval carcinoma line A431 (◆). The arrow indicates the concentration (2 μM) used in long-term exposure experiments. C, comparison of cell-free telomerase inhibition (TRAP) (◆) versus acute cytotoxicity against 21NT breast cancer cells (■). Values represent mean ± S.D.

There was no inhibition of the PCR at concentrations of up to 10 μM.

A key goal in the development of "second-generation" G-quadruplex-interactive telomerase inhibitors is to progress compounds not only with improved potency in the TRAP but also relatively low acute (presumed to represent nontelomerase specific) cytotoxicity (Neidle and Kelland, 1999; Kelland, 2001). Notably, BRACO19 was shown to induce acute cytotoxicity against a range of human tumor cell lines but at concentrations only above 5 μM. Individual IC_{50} values in micromolar range were A2780, 10; A2780cisR, 10; CH1, 10.1; CH1cisR, 10.3; SKOV-3, 13; and A431, 13 (and 21NT, 8) (mean IC_{50} of 10.6 $\pm$ 0.7 μM). This wide differential between enzyme inhibition and acute cell kill is illustrated for the 21NT breast cancer cell line (Fig. 2C); a differential of 70-fold is apparent at the respective 50% inhibitory levels.

Consequently, this permitted the study of BRACO19 in long-term exposure experiments in vitro at a concentration of 2 μM, well above that required for complete enzyme inhibition in the TRAP but well below that causing any acute cytotoxicity. Initial experiments focused on the 21NT breast cancer cell line (a line possessing relatively short telomeres; Cuthbert et al., 1999) because findings with dominant-negative hTERT (Hahn et al., 1999; Zhang et al., 1999) indicated that, as predicted by the telomere erosion model, cell kill or senescence should occur earlier in cells with short telomeres. However, it should be noted that some recent data show that telomerase inhibition may lead to loss of cell viability without measurable telomere erosion and be independent of initial telomere length (Kim et al., 2001; Saretzki et al., 2001). Exposure of 21NT cells to twice-weekly addition of 2 μM BRACO19 caused an approximately 50% reduction in cell growth at 8 days with almost complete inhibition by day 22 (Fig. 3A). Similar growth reduction effects were observed in another tumor cell line, A431, possessing relatively short telomeres, at 4 weeks post-treatment (data not shown). Notably, under the same exposure conditions, no growth inhibition of SKOV-3 cells (that possess relatively long telomeres) occurred over 5 weeks. The cessation in growth of BRACO19-exposed 21NT cells was not accompanied by any significant reduction in telomere length up to day 15 (by using a slot blot method to detect $TTAGGG_4$ repeats) in comparison with water controls (Fig. 3B). However, cellular telomerase activity was reduced at day 8 by approximately 50% (compare lane 7 with water control in lane 5) and was more marked by day 15, with approximately 90% reduction (compare lane 11 with water control in lane 9) (Fig. 3C). Note, there were insufficient cell numbers at day 22 to carry out telomere length or telomerase activity measurements. Finally, the cessation in cell growth of BRACO19-exposed 21NT cells was accompanied by a significant increase in cells staining positive for β-galactosidase, a biomarker of senescence, on days 15 and 22 (Fig. 3D).

The promising in vitro antitumor properties of BRACO19 were then extended into an initial investigation of antitumor efficacy, in vivo. The maximum tolerated dose of BRACO19 by single intraperitoneal injection was 5 mg/kg. Because the growth of 21NT cells in vivo as subcutaneous xenografts was both very slow and erratic, mice bearing A431 carcinoma xenografts were used. Drug treatments did not begin until tumors were palpable (6–8 mm diameter). In accordance with the possible clinical protocols for telomerase inhibitors (Kelland, 2001), BRACO19 was administered over prolonged periods (daily each weekday) either as a single agent or post-cytotoxic chemotherapy (paclitaxel) by using a nontoxic dose of 2 mg/kg/dose. Results are shown in Fig. 4 and reveal that BRACO19 when administered as a single agent (days 0–3, 6–10, and 13–17) did not induce significant antitumor activity in comparison with vehicle controls. However, the addition of BRACO19 (on days 13–17, 20–24, 27–31, and 34–38) after three doses of paclitaxel (25 mg/kg, on days 0, 4, and 8) induced a significant increase ($P < 0.05$) in antitumor effect in comparison with that observed with paclitaxel alone, on days 23, 27, 30, and 35. The time taken for tumors to reach 10 times their starting volume was extended by 10 days in the paclitaxel plus BRACO19 group (36 days) in comparison with the paclitaxel alone group (26 days). No toxicity or body weight loss was observed in either of the BRACO19-treated groups.

Discussion

Telomerase represents an attractive new target for cancer chemotherapy. The trisubstituted acridine BRACO19 represents a significant advance in the development of small-molecule G-quadruplex-interactive telomerase inhibitors. The existence of telomeric G-quadruplex DNA in vivo has recently been reported using *S. lemnae* macronuclei (Schaffitzel et al., 2001).

Although a number of G-quadruplex-interactive compounds have been described previously (Wheelhouse et al., 1998), including a number from our own studies (Sun et al., 1997; Perry et al., 1998a,b, 1999b; Harrison et al., 1999), these generally suffer from not only being particularly potent telomerase inhibitors (typical 50% inhibition values in the TRAP of 5–20 μM) but also exhibit acute cytotoxicity at similar concentrations to those required to inhibit the enzyme. We have also recently described a potent pentacyclic acridine-based G-quadruplex-interactive telomerase inhibitor (50% inhibition in the TRAP of 330 nM; Gowan et al., 2001). Recently, a series of potent (5–100 nM in the TRAP assay) ethidium- and dibenzophenathroline-based and a macrocyclic oxazole molecule (telomestatin; Shin-ya et al., 2001) telomerase inhibitor have also been described but no whole cell data were reported (Koeppel et al., 2001;Mergny et al., 2001). There have also been reports of relatively nonpotent small-molecule inhibitors of telomerase such as known reverse transcriptase inhibitors (e.g., zidovudine; Strahl and Blackburn, 1996), compounds of unknown mechanism of action (e.g., isothiazolones; Hayakawa et al., 1999), and the rhodocyanine FJ5002 (Naasani et al., 1999). Finally, a nonnucleosidic, non–G-quadruplex-interactive telomerase inhibitor (50% inhibition in the TRAP of 93 nM), BIBR1532, has recently been described (Damm et al., 2001).

We hypothesized that the narrow window between telomerase inhibition and acute cytotoxicity for the first-generation G-quadruplex-interactive agents was due to low relative binding affinities for quadruplex versus duplex DNA. Molecular modeling studies with bisubstituted acridine-based compounds (Harrison et al., 1999) revealed that the addition of a third substituent in the 9 position is predicted to lie in a third groove of the quadruplex. The resulting 9-dimethylamine anilino compound, BRACO19, was shown to possess much higher relative binding to quadruplex DNA versus duplex

DNA (25-fold) in comparison with the corresponding bisubstituted homolog (BSU-6048; 0.9-fold) (Read et al., 2001). Moreover, BRACO19 is 45-fold more potent than BSU-6048 in the TRAP and approximately 4-fold less cytotoxic in the acute SRB growth inhibition assay. We have also observed nuclear localization of BRACO19 after 30-min exposure of cells to 2 μM (data not shown). Further supportive, but indirect, evidence of a mechanism of telomerase inhibition by

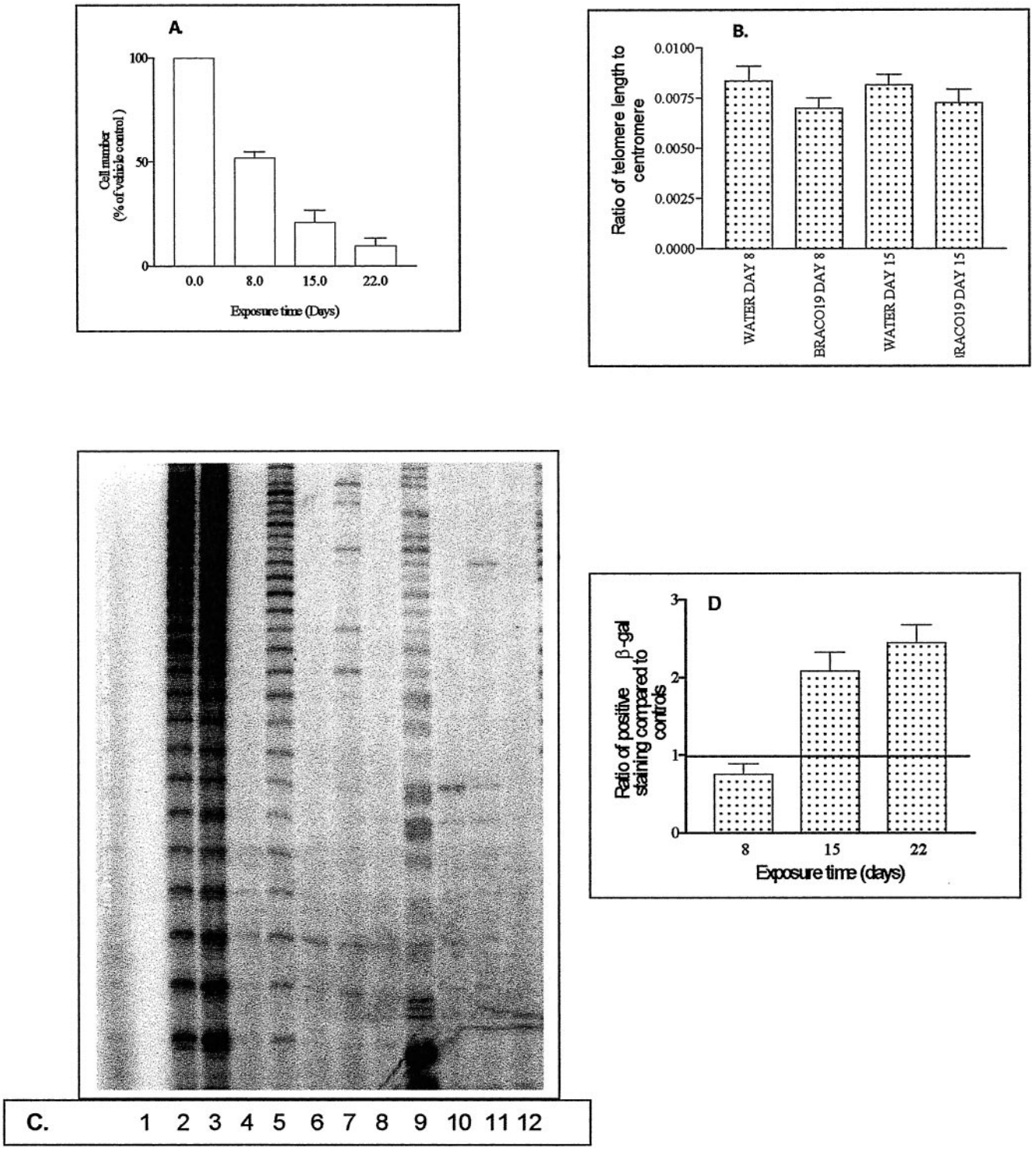


Fig. 3. Effect of long-term exposure of 21NT breast cancer cells to BRACO19 (2 μM) or an equivalent volume of water, twice weekly for 3 weeks. A, cell number expressed as a percentage relative to water controls. Control and treated flasks were passaged weekly and reseeded with 1.25×10^5 cells until cell numbers were too low. Values are mean ± S.D. B, telomere length (expressed relative to a centromere probe to normalize for loading). Values are mean ± S.D. C, cellular telomerase activity as assessed by TRAP. Lane 1, lysis buffer; lane 2, 20 ng of A2780 extract; lanes 3 and 4, 40 ng of A2780 extract; lanes 5 and 6, 40 ng of 21NT water on day 8; lanes 7 and 8, 40 ng of 21NT BRACO19 on day 8; lanes 9 and 10 40 ng of 21NT water on day 15; and lanes 11 and 12, 40 ng of 21NT BRACO19 on day 15. Lanes 4, 6, 8, 10, and 12 were all heat inactivated. D, β-galactosidase staining expressed relative to water controls. Values represent mean ± S.D.

BRACO19 involving stabilization of G-quadruplexes is provided by the TRAP gel of telomere products (Fig. 2A). The gel shows that even at relatively high concentrations of BRACO19 (0.5 μM, lane 5), the first three to four telomerase-induced hexanucleotide repeats are added, thereby fitting with a model where three to four hexanucleotide repeats are required to be added to the oligonucleotide template before quadruplex formation is permissible. Similar effects have been reported for another porphyrin G-quadruplex-interactive inhibitor (Wheelhouse et al., 1998).

Whole cell-based studies with BRACO19 are only partially supportive of a model whereby gradual telomere erosion is required before the induction of cellular senescence and or cell death. In 21NT cells possessing relatively short telomeres (approximately 2–3 kb) a marked reduction in cell growth, concomitant with an increase in β-galactosidase-positive cells, was observed at 15 and 22 days after twice-weekly addition of BRACO19 at 2 μM. In addition, under the same exposure protocol, no effect on cell growth was observed in another cell line, SKOV3, exhibiting similar sensitivity to the acute cytotoxic effects of BRACO19, but possessing longer telomeres. However, the cessation in cell growth of 21NT cells was not associated with any detectable decrease in telomere length as measured by a slot blot method detecting $TTAGGG_4$ repeats. This may be because 21NT cells already possess sufficiently short telomeres such that short-term disruption of the telomere/telomerase machinery is sufficient to induce cell death and not be accompanied with a detectable change in telomere length by the slot blotting method. Moreover, recent data suggest that the shortest telomere, not average telomere length, may represent the critical determinant of cell viability (Hemann et al., 2001). In addition, in some models, it has been proposed that changes in telomere uncapping versus capping status may be as important as actual telomere length in determining cell survival or death (Marusic et al., 1997; Blackburn, 2000; Kim et al., 2001; Saretzki et al., 2001). Further study is required in additional cell lines to fully eludicidate relationships between G-quadruplex-based telomerase inhibitor-induced cellular effects and telomere length and direct effects on telomerase.

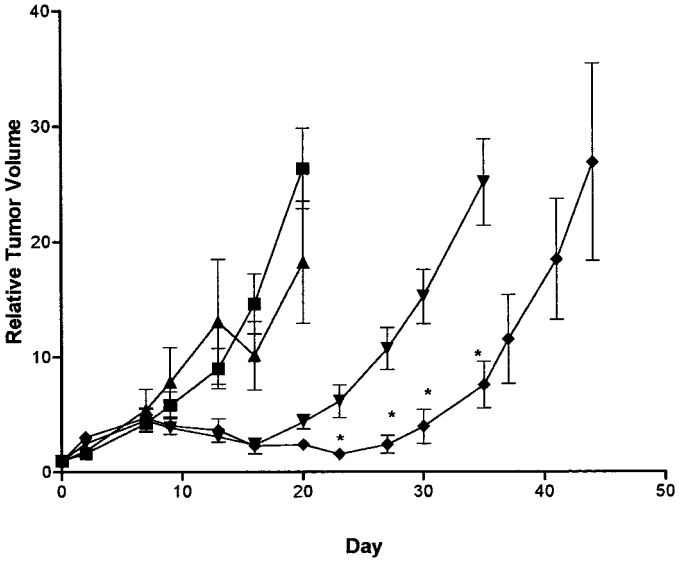


Fig. 4. Tumor growth curves (expressed a mean relative tumor volume) for mice bearing A431 human vulval carcinoma s.c. xenografts treated when tumor diameter reached 6 mm, with either vehicle (5% DMSO/95% water) (■), BRACO19 alone (2 mg/kg i.p. on days 0–3, 6–10, and 13–17) (▲), paclitaxel alone (25 mg/kg i.p. on days 0, 4 ,and 8) (▼), paclitaxel (25 mg/kg i.p.on days 0, 4, and 8), and BRACO19 (2 mg/kg i.p. on days 13–17, 20–24, 27–31, and 34–38) (◆). Values represent mean ± S.D. *, statistically significant difference from paclitaxel alone.

BRACO19 represents the first G-quadruplex-interactive telomerase inhibitor we are aware of to exhibit significant antitumor activity in vivo. When administered intraperitoneally to mice bearing palpable subcutaneous xenografts of the A431 human carcinoma, post-treatment with three doses of paclitaxel, BRACO19 (at a nontoxic dose) induced a significant additional antitumor effect to that observed with paclitaxel alone, extending regrowth to the 10-fold level by 10 days. Furthermore, BRACO19 did not cause any toxicity to the animals. The compound was inactive when administered singly, however. This reconciles with the concept that the 20 days permissible before control tumors had become too large for the animals from these groups to continue is insufficient for the telomere/telomerase disruption induced by BRACO19 to be manifest as a reduction in tumor size in comparison with controls. Furthermore, studies recently reported with a direct-acting telomerase inhibitor (BIBR1532) indicate that limited in vivo antitumor activity was only apparent after the long-term (>100 days) preexposure of cells to compound to shorten telomere length (Damm et al., 2001). In contrast, by targeting G-quadruplexes with BRACO19, significant antitumor activity post-paclitaxel treatment was apparent after only 4 weeks of daily, 5-day/week therapy.

A further combination study was attempted using xenografts derived from the SKOV-3 cell line (possessing longer telomeres) but were precluded because paclitaxel itself did not induce significant activity against this tumor. Further studies are required with BRACO19 to elucidate whether single agent activity might be achievable through administering the compound at the onset of tumor implantation in a more "chemopreventative" mode as advocated for antisense molecules directed at hTR (Herbert et al., 2001). Alternatively, greater activity might be obtainable through the use of continuous infusion via osmotic minipumps or continuous oral dosing. Initial pharmacokinetic studies with BRACO19 in mice showed that levels required to inhibit telomerase and cell growth in vitro (2 μM) are reached in plasma but maintained for only 30 min. It is also possible that improved antitumor efficacy will be achievable by combining the telomere/telomerase interactive BRACO19 with agents that induce DNA double-strand breaks, such as doxorubicin (Lee et al., 2001).

As for any new class of anticancer drug, the identification of appropriate pharmacodynamic markers of response represents an important adjunct to early clinical trials. Measurements of telomere length in tumors at the start of therapy may be useful to select antitelomerase treatment to tumors possessing relatively short telomeres but may not be appropriate to monitor response after treatment. Another pharmacodynamic endpoint revealed by our 21NT cell-based studies with BRACO19 is to monitor telomerase activity by TRAP because we observed a reduction in activity at days 8 and 15 post-exposure. This effect on telomerase has also been observed with a porphyrin-based G-quadruplex interactive telomerase inhibitor (Izbicka et al., 1999) and in our previous studies by using a pentacyclic acridine (Gowan et al., 2001). At present, the mechanism for this effect is unclear because

the quadruplex interactive strategy targets the telomere substrate rather than hTERT itself, but may be indicative of a close regulation between the telomere, telomere-associated proteins, and telomerase itself. However, the reduction in intracellular telomerase activity was not accompanied by a significant reduction in telomerase levels or effects on *c-myc* gene expression (as reported for the porphyrin-based G-quadruplex-interactive compound; Rangan et al., 2001) and is highly unlikely to be due to carryover of BRACO19 from the treated cellular extracts into the TRAP assay.

In summary, BRACO19 is a nanomolar small-molecule inhibitor of human telomerase in cell-free assays and, after 2 weeks of exposure at concentrations well below those causing acute cytotoxicity, a micromolar inhibitor of cell growth and inducer of senescence in a human breast cancer cell line. Moreover, proof of principle in terms of antitumor activity in vivo, albeit in combination with cytotoxic chemotherapy, is demonstrated for the first time with a G-quadruplex interactive strategy.

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References

Baumann P and Cech TR (2001) Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science (Wash DC)* **292:**1171–1175.
Bearss DJ, Hurley LH, and Von Hoff DD (2000) Telomere maintenance mechanisms as a target for drug development. *Oncogene* **19:**6632–6641.
Blackburn EH (2000) Telomere states and cell fates. *Nature (Lond)* **408:**53–56.
Blackburn EH (2001) Switching and signaling at the telomere. *Cell* **106:**661–673.
Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, and Reddel RR (1997) Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med* **3:**1271–1274.
Caprio V, Guyen B, Opoku-Boahen Y, Mann J, Gowan SM, Kelland LR, Read MA, and Neidle S (2000) A novel inhibitor of human telomerase derived from ^{10}H-indolo[3,2-b]quinoline. *Bioorg Med Chem Lett* **10:**2063–2066.
Clark GM, Osborne CK, Levitt D, Wu F, and Kim NW (1997) Telomerase activity and survival of patients with node-positive breast cancer. *J Natl Cancer Inst* **89:**1874–1871.
Cuthbert AP, Bond J, Trott DA, Gill S, Broni J, Marriott A, Khoudoli G, Parkinson EK, Cooper CS, and Newbold RF (1999) Telomerase repressor sequences of chromosome 3 and induction of permanent growth arrest in human breast cancer cells. *J Natl Cancer Inst* **91:**37–45.
Damm K, Hemmann U, Garin-Chesa P, Hauel N, Kauffmann I, Priepke H, Niestroj C, Daiber C, Enenkel B, Guilliard B, et al. (2001) A highly selective telomerase inhibitor limiting human cancer cell proliferation. *EMBO (Eur Mol Biol Organ) J* **20:**6958–6968.
Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA* **92:**9363–9367.
Dunham MA, Neumann AA, Fasching CL, and Reddel RR (2000) Telomere maintenance by recombination in human cells. *Nat Genet* **26:**447–450.
Fedoroff OY, Salazar M, Han H, Chemeris VV, Kerwin SM, and Hurley LH (1998) NMR-based model of a telomerase-inhibiting compound bound to G-quadruplex DNA. *Biochemistry* **37:**12367–12374.
Feng J, Funk WD, Wang S-S, Weinrich SL, Avilion AA, Chiu C-P, Adams RR, Chang E, Allsopp RC, Yu J, et al. (1995) The RNA component of human telomerase. *Science (Wash DC)* **269:**1236–1240.
Gowan SM, Heald R, Stevens MFG, and Kelland LR (2001) Potent inhibition of telomerase by small-molecule pentacyclic acridines capable of interacting with G-quadruplexes. *Mol Pharmacol* **60:**981–988.
Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, Beijersbergen RL, Knoll JHM, Meyerson M, and Weinberg RA (1999) Inhibition of telomerase limits the growth of human cancer cells. *Nat Med* **5:**1164–1170.
Hanahan D and Weinberg RA (2000) The hallmarks of cancer. *Cell* **100:**57–70.
Harley CB, Futcher AB, and Greider CW (1990) Telomeres shorten during ageing of human fibroblasts. *Nature (Lond)* **345:**458–460.
Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Bass MB, Arruda I, and Robinson MO (1997) A mammalian telomerase-associated protein. *Science (Wash DC)* **275:**973–977.
Harrison RJ, Gowan SM, Kelland LR, and Neidle S (1999) Human telomerase inhibition by substituted acridine derivatives. *Bioorg Med Chem Lett* **9:**2463–2468.
Hayakawa N, Nozawa K, Ogawa A, Kato N, Yoshida K, Akamatsu K-I, Tsuchiya M, Nagasaka A, and Yoshida S (1999) Isothiazolone derivatives selectively inhibit telomerase from human and rat cancer cells in vitro. *Biochemistry* **38:**11501–11507.
Hemann MT, Strong MA, Hao L-Y, and Greider CW (2001) The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* **107:**67–77.
Herbert BS, Pitts AE, Baker SI, Hamilton SE, Wright WE, Shay JW, and Corey DR (1999) Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci USA* **96:**14276–14281.
Herbert BS, Wright AC, Passons CM, Wright WE, Ali IU, Kopelovich L, and Shay JW (2001) Effects of chemopreventive and antitelomerase agents on the spontaneous immortalization of breast epithelial cells. *J Natl Cancer Inst* **93:**39–45.
Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA, and Shay JW (1995) Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* **1:**249–255.
Holt SE and Shay JW (1999) Role of telomerase in cellular proliferation and cancer. *J Cell Physiol* **180:**10–18.
Hsu H-L, Gilley D, Blackburn EH, and Chen DJ (1999) Ku is associated with the telomere in mammals. *Proc Natl Acad Sci USA* **96:**12454–12458.
Izbicka E, Wheelhouse RT, Raymond E, Davidson KK, Lawrence RA, Sun D, Windle B, Hurley LH, and Von Hoff DD (1999) Effects of cationic porphyrins as G-quadruplex interactive agents in human tumor cells. *Cancer Res* **59:**639–644.
Kelland LR (2000) Telomerase inhibitors targeting the vulnerable end of cancer? *Anticancer Drugs* **11:**503–513.
Kelland LR (2001) Telomerase biology and phase I trials. *Lancet Oncol* **2:**95–102.
Kerwin SM (2000) G-quadruplex DNAs a target for drug design. *Curr Pharm Des* **6:**441–471.
Kim S, Kaminker P, and Campisi J (2000) TIN2, a new regulator of telomere length in human cells. *Nat Genet* **23:**405–412.
Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL, and Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science (Wash DC)* **266:**2011–2015.
Kim MM, Rivera MA, Botchkina IN, Shalaby R, Thor AD, and Blackburn EH (2001) A low threshold level of expression of mutant-template telomerase RNA inhibits human tumor cell proliferation. *Proc Natl Acad Sci USA* **98:**7982–7987.
Koeppel F, Riou J-F, Laoui A, Mailliet P, Arimondo PB, Labit D, Petitgenet O, Helene C, and Mergny J-L (2001) Ethidium derivatives bind to G-quartets, inhibit telomerase and act as fluorescent probes for quadruplexes. *Nucleic Acids Res* **29:**1087–1096.
Lee K-H, Rudolph KL, Ju Y-J, Greenberg RA, Cannizzaro L, Chin L, Weiler SR, and DePinho RA (2001) Telomere dysfunction alters the chemotherapeutic profile of transformed cells. *Proc Natl Acad Sci USA* **98:**3381–3386.
Marusic L, Anton M, Tidy A, Wang P, Villeponteau B, and Bacchetti S (1997) Reprogramming of telomerase by expression of mutant telomerase RNA template in human cells leads to altered telomeres that correlate with reduced cell viability. *Mol Cell Biol* **17:**6394–6401.
Mergny J-L and Helene C (1998) G-Quadruplex DNA: a target for drug design. *Nat Med* **4:**1366–1367.
Mergny J-L, Lacrolx L, Teulado-Fichou M-P, Hounsou C, Guittat L, Hoarau M, Arimondo PB, Vigneron J-P, Lehn J-M, Riou J-F, et al. (2001) Telomerase inhibitors based on quadruplex ligands selected by a fluorescence assay. *Proc Natl Acad Sci USA* **98:**3062–3067.
Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Dickinson-Caddle S, Ziaugra L, Beijersbergen RL, Davidoff MJ, Liu Q, et al. (1997) *HEST2*, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* **90:**785–795.
Naasani I, Seimiya H, Yamori T, and Tsuruo T (1999) FJ5002: a potent telomerase inhibitor identified by exploiting the disease-oriented screening program with COMPARE analysis. *Cancer Res* **59:**4004–4011.
Neidle S and Kelland LR (1999) Telomerase as an anti-cancer target: current status and future prospects. *Anticancer Drug Des* **14:**341–347.
Perry PJ, Gowan SM, Read MA, Kelland LR, and Neidle S (1999a) Design, synthesis and evaluation of human telomerase inhibitors based upon a tetracyclic structural motif. *Anticancer Drug Des* **14:**373–382.
Perry PJ, Gowan SM, Reszka AP, Polucci P, Jenkins TC, Kelland LR, and Neidle S (1998a) 1,4- and 2,6-Disubstituted amidoanthracene-9,10-dione derivatives as inhibitors of human telomerase. *J Med Chem* **14:**3253–3260.
Perry PJ, Read MA, Davies RT, Gowan SM, Reszka AP, Wood AA, Kelland LR, and Neidle S (1999b) 2,7-Disubstituted amidofluorenone derivatives as inhibitors of human telomerase. *J Med Chem* **42:**2679–2684.
Perry PJ, Reszka AP, Wood AA, Read MA, Gowan SM, Dosanjh HS, Trent JO, Jenkins TC, Kelland LR, and Neidle S (1998b) Human telomerase inhibition by regioisomeric disubstituted amidoanthracene-9, 10-diones. *J Med Chem* **41:**4873–4884.
Pitts AE and Corey DR (1999) The telomerase challenge - an unusual problem in drug discovery. *Drug Discov Today* **4:**155–161.
Rangan A, Fedoroff OY, and Hurley LH (2001) Induction of duplex to G-quadruplex transition in the c-myc promoter region by a small molecule. *J Biol Chem* **276:**4640–4646.
Raynaud FI, Boxall FE, Goddard PM, Valenti M, Jones M, Murrer BA, Abrams M, and Kelland LR (1997) *cis*-Amminedichloro(2-methylpyridine) platinum(II) (AMD473), a novel sterically hindered platinum complex: in vivo activity, toxicology and pharmacokinetics in mice. *Clin Cancer Res* **3:**2063–2074.
Read MA, Harrison JR, Romagnoli B, Tanious FA, Gowan SM, Reszka AP, Wilson WD, Kelland LR, and Neidle S (2001) Structure-based design of selective and potent G-quadruplex telomerase inhibitors. *Proc Natl Acad Sci USA* **98:**4844–4849.
Read MA, Wood AA, Harrison JR, Gowan SM, Kelland LR, Dosanjh HS, and Neidle S (1999) Molecular modeling studies on G-quadruplex complexes of telomerase inhibitors: structure-activity relationships. *J Med Chem* **42:**4538–4546.
Saretzki G, Ludwig A, von Zglinicki T, and Runnebaum IB (2001) Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells *Cancer Gene Ther* **8:**827–834.

Schaffitzel C, Berger I, Postberg J, Hanes J, Lipps HJ, and Pluckthun A (2001) In vitro generated antibodies specific for telomeric guanine-quadruplex DNA react with *Stylonychia lemnae* macronuclei. *Proc Natl Acad Sci USA* **98:**8572–8577.
Shin-ya K, Wierzba K, Matsuo K-I, Ohtani T, Yamada Y, Furihata K, Hayakawa Y, and Seto H (2001) Telomestatin, a novel telomerase inhibitor from *Streptomyces anulatus*. *J Am Chem Soc* **123:**1262–1263.
Smith S, Giriat I, Schmitt A and de Lange T (1998) Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science (Wash DC)* **282:**1484–1487.
van Steensel B and de Lange T (1997) Control of telomere length by the human telomeric protein TRF1. *Nature (Lond)* **385:**740–743.
van Steensel B, Smogorzewska A and de Lange T (1998) TRF2 protects human telomeres from end-to-end fusions. *Cell* **92:**401–413.
Strahl C and Blackburn EH (1996) Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. *Mol Cell Biol* **16:**53–65.
Sun D, Thompson B, Cathers BE, Salazar M, Kerwin SM, Trent JO, Jenkins TC, Neidle S, and Hurley LH (1997) Inhibition of human telomerase by a G-quadruplex-interactive compound. *J Med Chem* **40:**2113–2116.
Wheelhouse RT, Sun D, Han H, Han FX, and Hurley LH (1998) Cationic porphyrins as telomerase inhibitors: the interaction of tetra-(N-methyl-4-pyridyl)porphine with quadruplex DNA. *J Am Chem Soc* **120:**3261–3262.
Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, Embleton J, Newell D, Raymond R, Stables J, et al. (1999) United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia. *Br J Cancer* **77:**1–10.
Zahler AM, Williamson JR, Cech TR, and Prescott DM (1991) Inhibition of telomerase by G-quartet DNA structures. *Nature (Lond)* **350:**718–720.
Zhang L, Mar V, Zhou W, Harrington L, and Robinson MO (1999) Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev* **13:**2388–2399.

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